12-2013

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Comments
This is a mansuscript of an article published as Nteeba, J., J. W. Ross, J. W. Perfield Ii, and A. F. Keating. "High fat diet induced obesity alters ovarian phosphatidylinositol-3 kinase signaling gene expression." *Reproductive toxicology* 42 (2013): 68-77. doi: 10.1016/j.reprotox.2013.07.026. Posted with permission.

Published in final edited form as:
Reprod Toxicol. 2013 December ; 42: . doi:10.1016/j.reprotox.2013.07.026.

High fat diet induced obesity alters ovarian phosphatidylinositol-3 kinase signaling gene expression

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Abstract

Insulin regulates ovarian phosphatidylinositol-3-kinase (PI3K) signaling, important for primordial follicle viability and growth activation. This study investigated diet-induced obesity impacts on: 1) insulin receptor (*Insr*) and insulin receptor substrate 1 (*Irs1*); 2) PI3K components (Kit ligand (*Kitlg),* kit (*c-Kit),* protein kinase B alpha (*Akt1)* and forkhead transcription factor subfamily 3 (*Foxo3a)*); 3) xenobiotic biotransformation (microsomal epoxide hydrolase *(Ephx1*), Cytochrome P450 isoform 2E1 (*Cyp2e1*), Glutathione *S*-transferase (*Gst*) isoforms mu (*Gstm)* and pi (*Gstp))* and 4) microRNA's 184, 205, 103 and 21 gene expression. INSR, GSTM and GSTP protein levels were also measured. Obese mouse ovaries had decreased *Irs1, Foxo3a, Cyp2e1, MiR-103,* and *MiR-21* but increased *Kitlg, Akt1*, and *miR-184* levels relative to lean littermates. These results support that diet-induced obesity potentially impairs ovarian function through aberrant gene expression.

Keywords

Obesity; phosphatidylinositol-3 kinase; ovary

1. Introduction

Globally, the prevalence of overweight and obesity is increasing, predisposing female to health hazards including diabetes, cardiovascular disease, cancer and compromised reproductive capacity [1-4]. 1.5 billion adults are overweight of whom 200 million men and 300 million women are obese. Further, approximately 43 million children (age 5-19) are overweight [5-10]. About 65% of the world's population live in countries where being overweight is more responsible for morbidity than underweight, and currently about 2.8 million adults globally die every year as a result of being overweight or obese [1]. Formerly considered a problem of developed countries, this epidemic is now dramatically on the rise in low- and middle-income countries which have had a long incidence of food insecurity,

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now squaring them up for a binary affliction of disease. While toiling with infectious disease and under-nutrition, they are also paradoxically experiencing a rapid upsurge in non-communicable disease risk factors including obesity and overweight [11-13].

The mammalian ovary is the female gonad responsible for gamete production, as well as the female sex steroids, 17β-estradiol and progesterone. The ovary contains a finite number of primordial follicles at the time of birth, which serve as the follicular pool. Once depleted, primordial follicles cannot be replaced [14-22]. Though this theory has been to a certain extent challenged in recent years [23-25], it remains that the number of primordial follicles declines over the female lifetime. This is because the process of follicular growth is an irreversible process; once follicles are recruited from the resting pool into the growing pool they will undergo cell death if not selected for further growth to ovulation [26-33]. Unlike the cyclic recruitment of follicles to ovulation, initial follicular activation is independent of the pituitary gonadotropins [17, 18, 34, 35]. The intrinsic ovarian mechanism(s) triggering individual follicle activation are not yet fully known, however, several lines of evidence have identified potential candidate factors including phosphatidylinositol-3 kinase (PI3K). An overall balance among dormancy, activation and death of primordial follicles is believed to be the determining factor which decides the female reproductive lifespan [29, 31, 36-38]. Any external factor that could accelerate follicle activation could hasten the rate at which the ovary become devoid of the follicular pool, thus threatening the reproductive potential of the female.

Obesity has been demonstrated to have detrimental effects on female reproductive function. Obese women have an increased likelihood to display signs of polycystic ovarian syndrome (PCOS), ovulation defects, reduced fecundity and poor quality oocytes [4, 39, 40]. There is also an association between obesity and an increased risk of birth defects, premature and still-births, and gestational diabetes [4, 41], however, the molecular mechanisms involved are still lacking. Obesity is a contributing factor for development of type 2 diabetics, characterized by elevated blood glucose and impaired insulin signaling [42]. Diet-induced obesity with concomitant diabetes is a stimulus that provokes alterations in insulin level and its subsequent sensitivity on its target tissues. It has been long postulated that obesity not only compromises insulin sensitivity in classical tissues like muscle and liver but also in other insulin responsive tissues like the ovary [42-44]. This theory has recently been challenged in a diet-induced obesity model, where ovaries from female mice fed on 60 % kcal of fat for 12 weeks maintained insulin sensitivity, despite that other classical tissues like muscle and liver became insulin resistant [45].

The ovary possesses both insulin and insulin-like growth factor 1 (IGF-1) receptors which can be directly stimulated by their hormone ligands. Insulin not only controls critical energy functions such as glucose and lipid metabolism but also plays a crucial role in reproductive function. Insulin receptor (INSR) is a heterodimer comprised of two alpha subunits and two beta subunits [46, 47]. Insulin binds to the alpha subunits which activates the insulin receptor tyrosine kinase in the beta subunits, with subsequent auto phosphorylation and recruitment of different substrate adaptors such as the Insulin receptor substrate (IRS 1-4) family of proteins. Tyrosine phosphorylated IRS then display binding sites for numerous signaling partners including the PI3K/Protein kinase B (PKB/AKT) signaling pathway. Defects in IRS, more specifically IRS1 and IRS2, have been implicated in female infertility and rodent models with such defects have been found to display both ovarian and hypothalamic dysfunction [48-50].

Several lines of evidence have identified the importance of PI3K signaling in ovarian function [51]. Once PI3K is activated, AKT is recruited to the cell membrane where it becomes phosphorylated (pAKT), and mediates many downstream events [52-54]. PI3K/

AKT has been implicated in regulating follicular activation [36, 38] recruitment, survival [29, 31], and development throughout the female reproductive lifespan [31, 51, 55-57]. PI3K also plays a role in proliferation and differentiation of granulosa cells (GC) in response to gonadotropins [29, 58] and it has been associated with ovarian carcinoma [31, 37, 38, 51, 55, 57-60]. In rodent models, $Akt^{-/-}$ mice were found to have reduced primordial follicle viability [57]. AKT has the ability to phosphorylate and inactivate several targets including forkhead transcription factors (FoxO). FoxO3 is critical for early stages of follicular growth [55] and it has been reported to trigger apoptosis through either up-regulation of genes necessary for cell death or down-regulation of anti-apoptotic genes [61-64]. $FOXO3^{-/-}$ mice had global primordial follicle activation by postnatal day 14, leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility [55] while in $FOXO3^{OE}$ mice, no activation of primordial follicle pool was observed [65].

Insulin-mediated PI3K activation has been also suggested to regulate xenobiotic biotransformation genes [66]. Inhibition of ovarian PI3K increases both mRNA and protein levels of *microsomal epoxide hydrolase* (*Ephx1*)[67] *Glutathione S-transferase Pi* (*Gstp*) [68] and *Glutathione S-transferase Mu 1* (*Gstm*) [69] suggesting that these enzymes could be downstream members of the PI3K signaling pathway. GSTP and GSTM are members of the GST protein family involved in phase II metabolism of xenobiotic compounds by converting a variety of electrophilic and hydrophobic compounds into more soluble, easily excretable compounds through catalyzing their conjunction with glutathione (GSH) [70]. EPHX1 and *Cytochrome P450 isoform 2E1* (CYP 2E1) ensure the rapid detoxification of epoxides generated during the oxidative metabolism of xenobiotics, thus providing cellular protection against free radical and carcinogenic compounds [66, 71-73]. Any alteration in expression patterns of genes that encode for ovarian chemical biotranformation enzymes can pose a risk for the onset of ovarian dysfunction. This is because exposure to a number of chemical classes can destroy follicles of all types threatening the reproductive potential of exposed females through accelerated premature ovarian insufficiency, premature ovarian failure (menopause) and other associated health problems [22, 67, 74-77].

Several genes that are components of the PI3K pathway also are regulated by MicroRNAs (miR's) [78-80]. MiR's are small (19 – 25 bp) non-coding RNA that can positively or negatively regulate gene expression [81-85]. It is known that miR-21 inhibits phosphatase and tensin homolog (PTEN), an antagonist of PI3K [86, 87]. *In vivo* loss of miR-21 has been reported to increase ovarian apoptosis and as well compromise ovulation rates in rodent models [86, 88]. MiR-184 is believed to play a critical role in development as well as a mediator of apoptosis [79]. Up regulation of miR-184 can interfere with AKT action, repressing PI3K action [78, 85]. Also, miR-103 has been implicated in insulin sensitivity [89]. Thus, miR's may mediate the response to insulin signaling, through the PI3K pathway.

In summary, obesity results in reproductive dysfunction and an increase in negative consequences for offspring, and insulin signaling is impaired in obesity. Additionally, insulin can activate PI3K signaling, which is critical for controlling the rate of activation of primordial follicles, and is an upstream regulator of xenobiotic metabolism gene expression. There remains a dearth of knowledge regarding whether obesity can influence ovarian xenobiotic metabolism, thus we hypothesized that obesity caused by a high fat diet would alter ovarian PI3 kinase signaling with subsequent effects on genes encoding xenobiotic metabolism enzymes in female mice.

2. Materials and Methods

2.1. Reagents

2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N',N',N',N'-Tetramethyl-ethylenediamine (TEMED), Tris base, Tris HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Hanks' Balanced Salt Solution (without CaCl2, MgCl2, or MgSO4), DAPI nuclear stain and superscript III one-step RT-PCR System were obtained from Invitrogen Co. (Carlsbad, CA). miRNeasy Mini Kit, miScrip Reverse Transcription Kit, miScrip SYBR Green PCR Kit, RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit, TaqMan® microRNA Reverse Transcription Kit and QuantitectTM SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL). Custom designed primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University. Ponceau S was purchased from Fisher Scientific (Waltham, MA, USA). ECL plus chemiluminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK). Anti-GSTP and anti-GSTM antibodies were purchased from Millipore (Temecula, CA, USA). Anti-pAKT^{Ser473} and β-Actin antibodies were from Cell Signaling Technology and anti-insulin receptor (INSR) antibody was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies were obtained from EMD Millipore (Billerica, MA).

2.2. Animal and diets

Ovarian tissues were obtained from a study at the University of Missouri. The experimental protocols were approved and performed in accordance with the guidelines of the University of Missouri Institutional Animal Care and Use committee as previously described [90]. Briefly, twelve 6 weeks old C57Bl/6J female mice were randomized into two groups (n = 6 per group). The control group was fed a standard chow mice diet (Purina 5001; 4.5g/100g fat) while the treatment group was fed a high-fat diet (HFD; Research Diets D12492; 35g/100g fat) for approximately 7 months. The animals were housed at constant room temperature, 12h light:12h darkness cycle, diet and water were provided *ad libitum*. Body weight for the control and HFD mice were 24.8g ± 0.45 and 46.5g ± 2.0, respectively. Gonadal adipose tissue weight was 0.44g ± 0.05 and 3.74g ± 0.35 in the control and HFD mice, respectively [90]. After 7 months glucose tolerance testing confirmed that HFD-fed mice had elevated blood glucose, relative to their control-diet fed littermates [90]. There was no impact of HFD on plasma estradiol [90]. The HFD mice are subsequently referred to as obese, and their non-HFD fed littermates are referred to as lean.

2.3. Tissue collection

Animals were euthanized by CO_2 asphyxiation. Ovaries were removed, trimmed of excess fat, weighed and snap frozen for gene expression analysis or fixed in 4% paraformaldehyde for histological analysis.

2.4. RNA Isolation

Total ovarian RNA was isolated using Qiagen RNeasy® Mini Kit (n = 6 per dietary treatment). Briefly, ovaries were lysed and homogenized using a hand held homogenizer. The homogenate was then applied to a QIAshredder column placed in a collection tube and centrifuged at 16100 RCF for 2 minutes at room temperature. The flow through was then applied to an RNeasy Mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute kit.

RNA was eluted using 14 μl of RNase-free water and quantified using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE).

2.5. First Strand cDNA synthesis and quantitative Real-Time quantitative polymerase chain reaction (qRT-PCR)

Total RNA (0.5 μg) was reverse transcribed to cDNA using Invitrogen™ Superscript III Reverse Transcriptase according to the manufacturer's protocol. Diluted cDNA (2 μl; 1:20 dilution) were amplified on an Eppendorf Mastercycler using Quantitect™ SYBR Green PCR kit and primers specific for mouse Actb, Insr, Irs1, Kit Ligand (Kitlg), c-Kit, Akt1, Foxo3a, Cyp2e1, Ephx1, Gstp, and Gstm (See table 1 for primer sequences). The PCR cycling program consisted of a 15 min hold at 95 °C and 40 cycles of: denaturing for 15 at 95 °C, annealing for 15s at 58 °C and extension at 72 °C for 20s. Product melt conditions were determined using a temperature gradient from 72 °C to 99 °C with a 1 °C increase at each step. Three replicates of each sample (n = 6 per dietary treatment) were included. Statistical analysis was performed on the cycle numbers at which each sample reached a threshold level. The relative mRNA expression for each of the above genes was normalized using mouse *Actb* as a housekeeping gene and relative fold change calculated using the $2^{-\Delta\Delta CT}$ method. The results are presented as mean fold change ± standard error relative to the control group.

2.6. mirScript miRNA PCR array

Purification of miRNA-enriched fractions was performed using a miRNeasy Mini Kit according to manufacturer's protocol (n = 3 per dietary treatment). MiRNA-enriched fractions (250 ng) in a reverse-transcription reaction of 20 μl were converted to cDNA using miScrip Reverse Transcription Kit. cDNA was then diluted by adding 200 μl of RNase-free water, from which, 100 μl was used as template cDNA for real-time PCR quantification of multiple microRNAs using miScrip SYBR Green PCR Kit. Data analysis was performed using the web-based miScript miRNA PCR Array Data Analysis package. TaqMan® microRNA Reverse Transcription Kit was used to validate the expression of miR-21. The amount of target miRNA was normalized using RNU43 as a housekeeping miRNA.

2.7. Histological analysis of ovarian tissue

One ovary from each animal was fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned (5 μM) at the Iowa State University Veterinary Medicine Histopathology laboratory. Sections were mounted (3-4 per animal), and one section was stained with hematoxylin and eosin (H&E). Digital images were acquired with a Leica DMI300B Fluorescent Microscope.

2.8. Protein Isolation and Immunoblot analysis

Total ovarian protein was isolated and immunoblots performed as previously described 75. Briefly, ovaries were homogenized in ~ 200 μl of iced-cold extraction buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP40 and 1% Protease inhibitor, followed by centrifugation for 30 minutes at 9300 RCF at 4 °C. Protein concentration was determined using a standard BCA protocol. Emission absorbance values were detected with a λ = 560 nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-Tek^{R} Instruments Inc. Winooski, VT). Total protein (20 μg) was separated using 10% SDS-PAGE and electro-transfer of proteins from the gel to a nitrocellulose membrane was performed for 60 minutes at 100 V. The membranes were stained with Ponceau S to visualize the amount of total protein transferred in each lane. To reduce non-specific binding, membranes were pre-incubated overnight on a rocker at 4°C in a blocking buffer (5% non-fat dry milk, 5 M NaCl, 20 mM Tris-HCl, 0.15% Tween-20, pH 8).

Membranes were probed using specific primary antibodies; Rabbit Anti-INSRα (1:500); Rabbit anti-GSTP (1:200); and mouse Anti-ACTB (1:2000) diluted in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TTBS) overnight at 4 °C. Following washing for three times (10 min each) in TTBS, membranes were incubated at room temperature for 1 hour with HRP-conjugated suitable secondary antibodies (1:2000) against the primary antibodies. Membranes were washed three times in TTBS followed by a single wash in Tris-Buffered Saline (TBS). Autoradiograms were visualized on X-ray films in a dark room following 5 min incubation of membranes in ECLplus chemiluminescence detecting reagent. Densitometry of the appropriate band was quantified using Image J software (NCBI). Equal loading was confirmed by Ponceau S staining of membranes and protein expression was normalized to ACTB densitometry values.

2.9. Immunoflourescence staining

Two slides per animal were deparaffinized in citrol buffer and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with a primary antibody directed against $pAKT^{Ser473}$ (1:100) overnight at 4°C. After washing in 1% PBS, sections were incubated with a goat anti-rabbit secondary antibody conjugated to fluorescein for 1 h. Slides were counterstained with DAPI nuclear stain for 5 min. Images were taken using a Leica fluorescent microscope. Analysis of $pAKT^{Ser473}$ protein level in small oocytes (pre-antral follicles), large oocytes (antral follicles) and theca cells of antral follicles was performed using ImageJ software (NCBI). Mean densitometry value was divided by the cellular area measured.

2.10. Statistical analysis

Statistical analysis was performed using the unpaired t-test function of GraphPad Prism 5.5 software with a statistical significance level set at $P < 0.05$. $P < 0.1$ was considered a trend for a difference between treatments.

3. Results

3.1. Effect of obesity on ovarian size and weight

There was no impact of HFD on ovarian weight ($P > 0.05$) ovarian weight (Figure 1A) yet the ovaries appeared visually smaller relative to their lean littermates (Figure 1B and 1C).

3.2. Effect of obesity on ovarian insulin signaling members

Obesity decreased mRNA level of the gene encoding the *Irs1* ($P < 0.05$) with a trend for a decrease in *Insr* ($P = 0.08$; Figure 2A). Additionally there was a strong trend ($P = 0.06$) for decreased INSR protein levels in ovaries of obese female mice compared to lean mice (Figure 2B and 2C).

3.3. Effect of obesity on ovarian PI3K signaling

Relative to lean ovaries, obesity increased the mRNA levels of *Kitlg* by 0.6-fold ($P < 0.05$) and there was a trend for increased mRNA level of the KITLG receptor, *c-Kit* of 0.4-fold ($P = 0.07$; Figure 3). Obesity also induced a 1.1-fold increase ($P < 0.05$) in mRNA levels of *Akt1*, with a concomitant 0.6-fold decrease ($P < 0.05$) in mRNA levels of *Foxo3a* relative to lean ovaries (Figure 3). $pAKT^{Ser473}$ protein was localized to the oocyte of pre-antral and antral follicles and also to the theca cells (Figure 4A,B). Obese females had a strong trend ($P = 0.056$) for decreased $pAKT^{Ser473}$ protein in the oocytes of pre-antral follicles (Figure 4C), with no impact observed in the antral follicle oocytes (Figure 4D). Interestingly, there was a

trend ($P = 0.09$) for increased $pAKT^{Ser473}$ protein in theca cells from obese females (Figure 4E).

3.4. Effect of obesity on ovarian expressed xenobiotic metabolism genes

There was decreased mRNA level for the gene encoding *Cyp2e1* ($P < 0.05$). In addition a trend ($P < 0.1$) for increased *Ephx1* and decreased *Gstp1* and *Gstm1* mRNA levels was observed (Figure 5A). No impact of obesity on GSTM protein was observed, however, there was a trend for decreased GSTP protein level ($P < 0.1$).

3.5. Effect of obesity on ovarian expression of miR-103, miR-21, miR-184 and miR-205

Ovaries from obese mice had decreased ($P < 0.05$) *miR-21*, and *miR-103* ($P < 0.05$), with a strong trend ($P = 0.06$) for increased *miR-205.* In contrast, obesity up-regulated ($P < 0.05$) *miR-184* levels (Figure 6A). The obesity-induced decrease in *miR-21* was validated using qRT-PCR ($P < 0.05$; Figure 6B).

4. Discussion

Several studies have highlighted a strong correlation between obesity, infertility and adverse reproductive health outcomes, however, the underlying mechanisms remain unclear [91-95], thus comprehension of the mechanisms by which obesity affects ovarian function is of significant relevance. We hypothesized that obesity could alter factors regulating both primordial follicular activation and xenobiotic biotransformation. The females in this study were approximately 8.5 months of age, and did not differ in levels of plasma estradiol [90].

Previous studies in humans and rat models have reported that hyperinsulinemia down-regulates ovarian *Insr* expression [96, 97]. Similar studies in insulin-resistant and hyperinsulinemic mice demonstrated altered duration of estrous cycles as well as aberrant distribution and morphology of ovarian follicles [42, 49, 98]. The metabolic effects of insulin mainly involve tissue-specific actions which result in changes in gene expression, protein phosphorylation and function of the INSR and its downstream adaptor proteins, IRS1-4 [99]. Phosphorylation of IRSs lead to activation of downstream mediators including the PI3K/AKT serine/threonine kinase [50]. There are several pathological effects associated with dysregulated PI3K pathway in the ovary. For instance, over activation of PI3K in oocytes has been associated with premature ovarian failure (POF), while, on the other hand, over activation of the pathway in granulosa cells is highly correlated with defects in follicle cyclic recruitment and ovulation and carcinogenesis derived from ovarian surface epithelium [29-31, 37, 58, 100].

In order to determine the molecular effects of obesity on ovarian function, we first investigated the effect of obesity on expression of genes encoding the insulin signaling members, INSR and IRS1, both of which are critical for the response to insulin. Obesity decreased the mRNA level of *Irs1* concomitant with a trend for a reduction in total ovarian *Insr* mRNA and protein expression. The effect of obesity on expression of insulin signaling members in other tissues is well reported but most ovarian studies have reported inconsistent results. For instance consistent with our findings, reductions in expression of INSR and IRS1 during obesity [101-103] or hyperinsulinemia [96, 97] have been reported, while in contrast, increased phosphorylation of both IRS1 and IRS2 with obesity have been also observed in other cell types [45, 96, 104-107]. Additionally, other studies have observed no effect of obesity on INSR [3, 42] or IRS1 [3, 108, 109]. Taken together, these results implicate the effect of obesity on insulin signaling members to be a complex mechanism that may be species-, tissue- and/or time-specific.

Following these observations we postulated the insulin-mediated PI3K/AKT pathway could be compromised. To our surprise our results demonstrated that reduction in mRNA encoding insulin signaling factors did not inactivate the PI3K/AKT pathway in ovaries of obese mice. Obese mice had increased *Akt1* mRNA levels with a concomitant decrease in *Foxo3a* mRNA compared to lean mice. Several other studies show that insulin could have a tissue-isoform and species specific differential effect on *Akt* isoforms [53, 110]. In obese rodent models, insulin-stimulated AKT1 activity was reported to decrease in muscle and adipose tissue but increased in liver, in contrast, AKT2 activity was decreased in muscle and liver but increased in adipose tissue [53]. Thus, our data indicate that ovarian AKT1 responds in a similar fashion to hepatic tissue in the face of changing insulin levels. Whether these changes are direct interactions between the IRS or involve other signaling mediators is difficult to surmise from the current data.

Since obesity mildly reduced expression of *Insr* and *Irs1* in mice ovaries while the PI3K signaling pathway is markedly up-regulated compared to lean mice in our study, we asked what other insulin-independent pathways involved in activation of the ovarian PI3K/AKT pathway were altered in the obese mouse ovary. Stem cell growth factor receptor (c-KIT), a receptor protein tyrosine kinase is expressed by the oocyte [111, 112] and its ligand, Kit Ligand (KITLG; also called Stem cell factor or Steel factor), is expressed by granulosa cells [113]. Several lines of evidence have indicated that KITLG/c-KIT signaling is essential for oocyte viability and survival and that this pro-survival pathway also regulates follicle recruitment [114-116]. Once c-KIT is bound by KITLG the PI3K pathway is activated [36-38, 55, 56, 59]. Based on those previous reports and our data we asked if HFD-induced obesity affected the ovarian mRNA level of genes encoding c-KIT and KITLG. Our data shows that obesity increased *Kitlg* mRNA levels with a trend for increased mRNA encoding its receptor, c-*Kit*. To the best of our knowledge this is the first study to demonstrate that HFD-induced obesity can alter the expression of the c-*Kit* ligand in murine ovaries. In oocytes of cultured neonatal mouse and rat ovaries, it has been demonstrated that KITLG leads to phosphorylation of FOXO3a through the action of pAKT [36]. Based on the results presented herein hyperactivation of KITLG/c-KIT-PI3K/AKT could be a potential mechanism underlying obesity related infertility problems. It is known that hyperstimulation of primordial follicles into the growing follicular pool leads to their eventual destruction [55, 57, 59]. Over-activation of the KITLG/c-KIT dependent PI3K/AKT pathway could imply that in obese female mice, there is over stimulation of primordial follicles into the growing follicular pool; a scenario that could accelerate the rate at which mice become infertile, although it should be noted that follicle numbers were not classified in our study. Interestingly, we also noted a trend for increased theca cell $pAKT^{Ser473}$, and it is known that the interaction between KITLG and c-KIT has a regulatory role in steroidogenesis in rat granulosa cells [117].

Several studies have demonstrated that insulin and other growth factors regulate xenobiotic chemical metabolizing enzyme gene expression, including cytochromes p450, GSTs and EPHX1 through several kinases including PI3K/AKT signaling pathway [66, 118, 119]. The ovary has the capacity to metabolize ovotoxic compounds [60, 67, 68, 75, 76, 120-122], however to date, there is a dearth of literature examining potential effects of obesity on expression of xenobiotic metabolizing enzymes in the ovary. Since diet-induced obesity can alter insulin levels and/or sensitivity, we hypothesized that obesity may alter the ovarian expression of ovotoxicant metabolizing enzymes genes. Previous studies have demonstrated that diabetes is strongly associated with increased hepatic *Cyp2e1* [123] and on the other hand decreased *Ephx1* [124]. Also, *Cyp2e1* mRNA expression is decreased in a dose dependent manner by insulin in rat cultured hepatocytes [123, 125, 126]. PI3K inhibitors such as wortmannin and LY294002 were shown to reverse the insulin-mediated down-regulation of *Cyp2e1* mRNA levels [127]. Furthermore, in cultured neonatal ovaries,

inhibition of PI3K signaling using LY294002 resulted in increased mRNA and protein levels of *Ephx1, Gstp* and *Gstm* [67-69]. In the present study decreased mRNA encoding *Cyp2e1* was observed in ovaries of obese mice compared to the lean mice. In contrast to *Cyp2e1*, there was a trend for increased *Ephx1* mRNA level. This differential effect of a physiological paradigm on ovarian *Cyp2e1* and *Ephx1* expression has been previously reported [76, 128]. Studies on the GSTs have reported both increased and decreased gene expression during diabetes [66]. In rat cultured hepatocytes, neither insulin nor glucagon affected the mRNA levels of *Gstm* while, on the other hand, glucagon reduced *Gstp* mRNA levels [129]. In the current study there was a trend for decreased *Gstp* mRNA and protein level in the obese ovary. These results suggest that obese females may have altered xenobiotic metabolism and therefore if such females are exposed to ovotoxicants, the rate at which they would approach premature ovarian insufficiency and failure would be accelerated. Further still, these results suggest that obese females may have increased risks from exposure to carcinogens and teratogens which could also potentially explain the increased rates of miscarriage and increased offspring birth defects in obese mothers.

PI3K signaling can be regulated through posttranscriptional gene silencing by the action of miRs [78, 80, 89, 130]. In order to evaluate if obesity had any effect on miR's that could at least partially explain the effects observed on PI3K signaling, levels of *miR-103, miR-21, miR-184* and *miR-205* were measured. It was found that the ovaries from obese mice had decreased *miR-21* and *miR-103*; however, *miR-184* levels were increased. Recent reports have indicated that *miR-21* expression is important for regulation of apoptosis [131, 132]. Decreased expression of *miR-21* expression has been reported to increase cell apoptosis in a variety of cell culture systems including the granulosa cells from mouse pre-ovulatory follicles both *in vivo* and *in vitro* [86]. In addition, *miR-21* has been identified as promoting follicular cell survival during ovulation and *miR-21* inhibition also has been reported to reduce ovulatory rates [86]. Although many different cell types undergo apoptosis in response to inhibition of *miR-21* action, the *miR-21* targets implicated vary widely for different cells and the mechanism by which *miR-21* suppresses apoptosis in GCs remain to be identified [133-135].

miR-184 has been shown to act as a physiological suppressor of general secretory activity of progesterone and estradiol [135, 136]. Additionally, *miR-184* is believed to play a critical role in development as well as being a mediator of apoptosis. Up-regulation of *miR-184* has been reported to interfere with the ability of *miR-205* to lead to repression of AKT signaling [78]. Though in this study we observed an elevated level of *miR-184* with a trend for a decrease in *miR-205* level, there was an up-regulation of *Akt1* mRNA levels together with a subsequent decrease in *Foxo3a* mRNA levels thus indicating that *Akt1* activation is not being compromised by *miR-205* action. Several studies from both human and rodent models have reported that *miR-103* is up-regulated during obesity [89, 137, 138]. It has been further suggested that silencing *miR-103* improves insulin sensitivity in adipocytes mainly through increased caveolin-1 expression, which in turn leads to stabilization of the INSR and thus enhancing insulin signaling [89]. In contrast to the above reports *miR-103* expression has also been found to be down-regulated in the mouse model of genetic insulin resistance and obesity (ob/ob mice) [139]. This is consistent with our findings, where obesity decreased *miR-103* levels in mice ovaries. These results further confirm miRNA cell and tissue specific actions [140].

5. Conclusions

In summary, the data presented herein, though preliminary in nature, demonstrate perturbations to ovarian PI3K signaling caused by obesity in females. Under basal conditions, AKT1 is positively regulated by both KITLG and IRS1. In addition, AKT1 is

positively regulated by miR21 and negatively regulated by miR184. Our data collectively indicates that obesity decreases IRS1 levels but increases KITLG which can then result in increased AKT1 levels. Although miR21 is decreased, which should result in decreased AKT1, we propose that increased levels of miR184 may counteract decreased miR21 to result in AKT1 activation (summarized in Figure 7). One consequence of obesity-induced altered PI3K includes changes in the downstream components *Akt1* and *Foxo3a* that may alter the rate of primordial follicle activation, leading to fertility problems. Another result of altered PI3K observed is that expression of major xenobiotic biotransformation enzymes that are important in the ovarian response to ovotoxicant exposure, are changed. Thus, obesity may have negative consequences for follicle activation and oocyte viability, as well as altering how the ovary responds to chemical exposures. All of these scenarios can lead to impairment of ovarian function, and may at least partially explain why reproduction is compromised in obese females.

Acknowledgments

The project described was supported by the National Institutes of Environmental Health Sciences [R00ES016818] to A.F.K. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

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Highlights

- High fat feeding increased phosphatidylinositol-3 kinase signaling
- Obese mice had altered xenobiotic metabolism gene expression
- Obesity induced altered microRNA expression

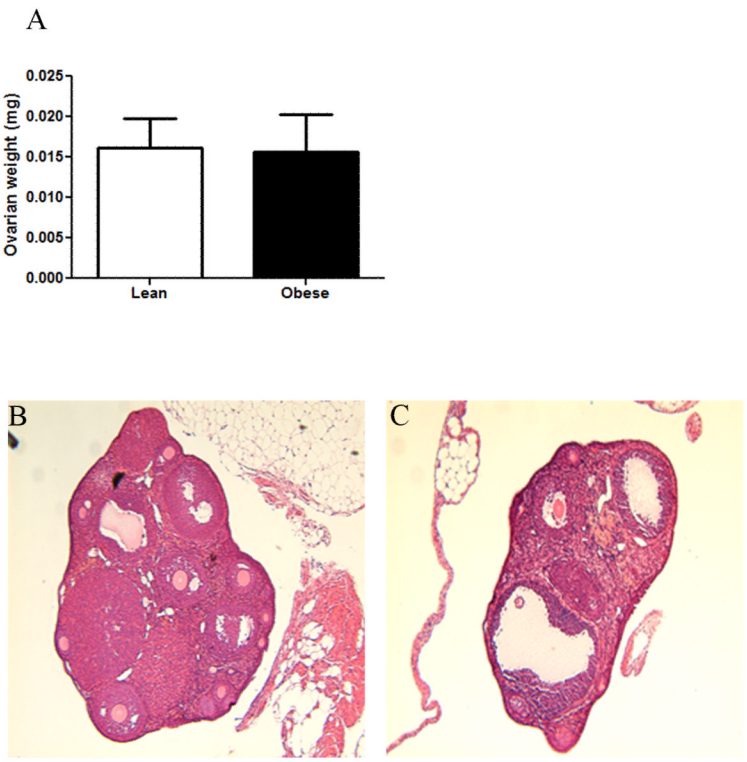

Figure 1. Obesity does not alter ovarian weight in mice

Six weeks old C57Bl/6J female mice (n = 6 per treatment group) were fed either a standard chow mice diet or a high-fat diet for approximately 7 months and were euthanized by CO_2 asphyxiation. Ovaries were removed, trimmed of excess fat and weighed. (A) Ovarian weight; (B) lean and (C) obese ovarian sections stained with hematoxylin and eosin (H&E).

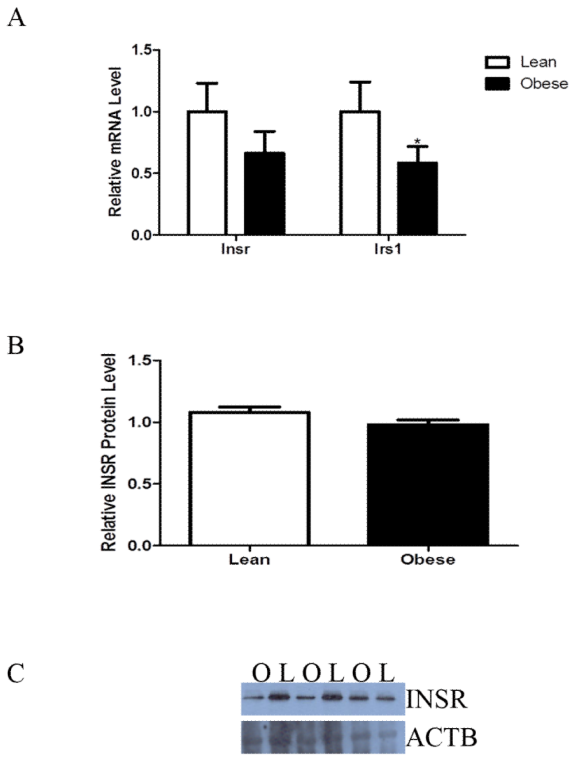

Figure 2. Obesity decreases ovarian *Irs1* mRNA levels in mice

Ovaries were removed from lean and obese mice (n = 6 per treatment group). (A) RNA was isolated and *Insr* and *Irs1* mRNA levels were evaluated by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to *Actb*. (B) Total protein was isolated and Western blotting performed to measure INSR level. Values represent fold-change relative to a control value of 1 ± SE, normalized to ACTB. Different from control, $*P < 0.05$. (C) Representative Western blot for INSR and ACTB; Lean = L, Obese = O.

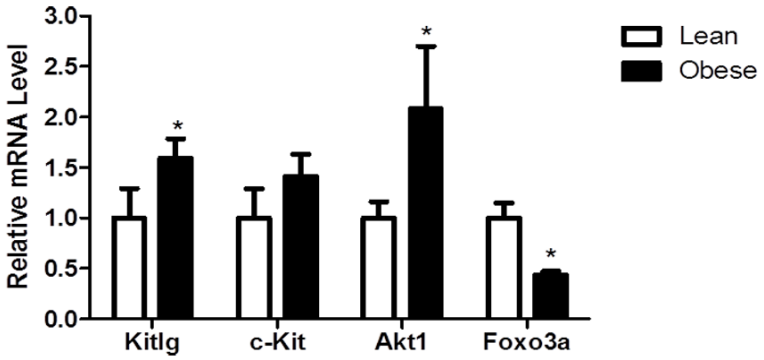

Figure 3. Obesity enhances ovarian KITLG/KIT-PI3K/AKT1 signaling pathway in mice

Ovaries were removed from lean and obese mice (n = 6 per treatment group). RNA was isolated and *Kitlg, cKit, Akt1* and *Foxo3a* mRNA levels were evaluated by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to *Actb*. Different from control, $*P < 0.05$.

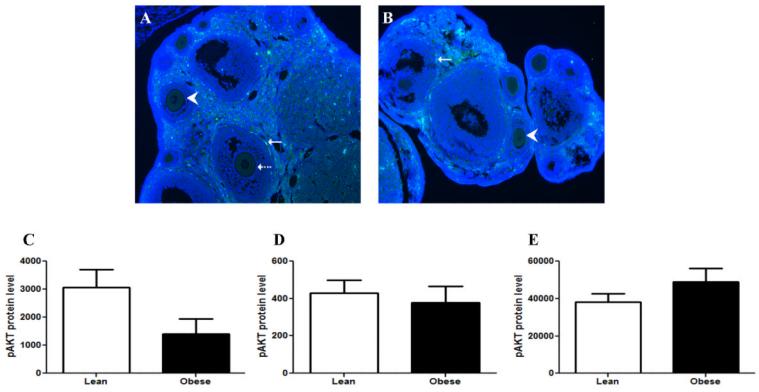

Figure 4. Altered pAKT^{Ser473} levels are observed in the ovaries of obese mice

Ovaries were removed from (A) lean and (B) obese mice, serially sectioned and mounted onto slides (n = 6 per treatment group). Immunoflourescence staining to detect pAKT^{Ser473} was performed, with counterstaining to detect the nucleus. Image J software was used to quantify levels of pAKT^{Ser473} in (C) small (pre-antral; indicated by arrowhead) and (D) large oocytes (antral; indicated by broken arrow) and (E) theca cells (indicated by unbroken arrow).

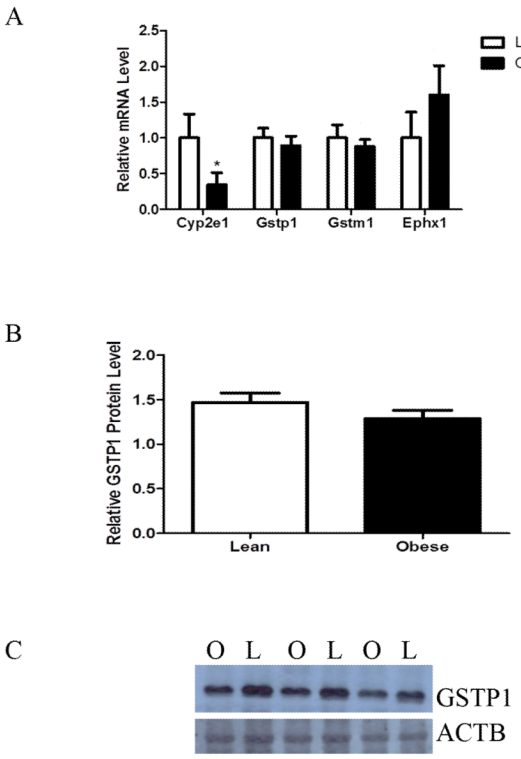

Figure 5. Obesity down-regulates ovarian *Cyp2e1* mRNA levels

Ovaries were removed from lean and obese mice and RNA isolated (n = 6 per treatment group). (A) *Cyp2e1*, *Ephx1, Gstp* and *Gstm* mRNA levels were measured by quantitative RT-PCR. Values represent fold-change relative to a control value of 1 ± SE, normalized to *Actb*. Different from control, $*P < 0.05$. (B) Western blotting was performed to determine any impact of obesity on GSTP. (C) Representative Western blot for GSTP and ACTB; Lean = L, Obese = O.

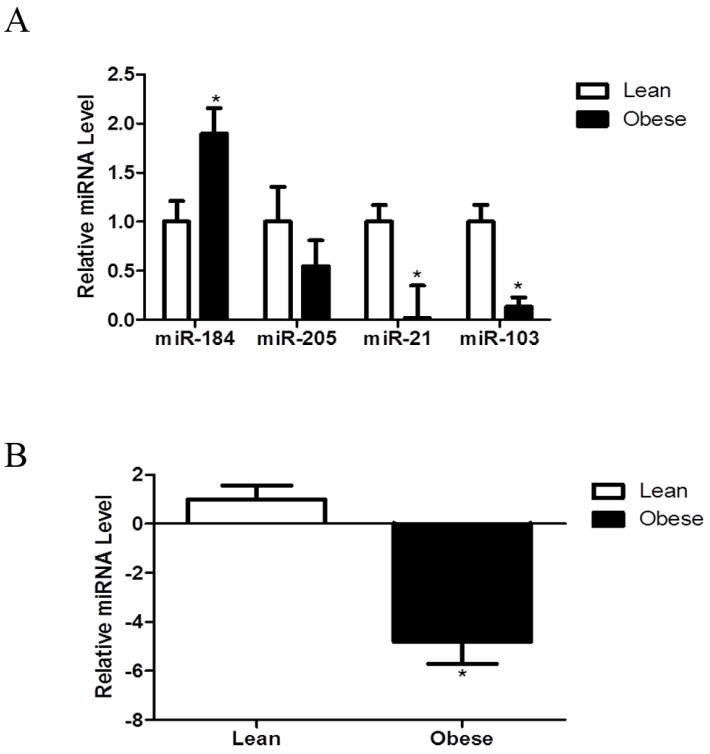

Figure 6. Obesity alters levels of ovarian miR

Ovaries were removed from lean and obese mice and RNA isolated (n = 3 per treatment group). (A) Enriched miR fractions were analyzed using a miR array. (B) qRT-PCR was performed on miR-21 to confirm the array data. Values represent fold-change relative to a control value of 1 ± SE, normalized to RAU43. Different from control, **P* < 0.05.

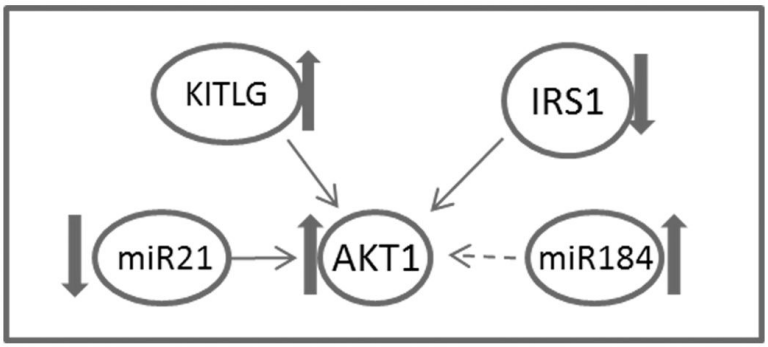

Figure 7. Proposed model of obesity effects on AKT1 signalinga

Under basal conditions, AKT1 is positively regulated by both KITLG and IRS1. In addition, AKT1 is positively regulated by miR21 and negatively regulated by miR184. Our data indicates that obesity decreases IRS1 levels but increases KITLG leading to increased AKT1 levels. The increased levels of miR184 may counteract the decreased miR21 in AKT1 activation. AKT regulates xenobiotic metabolism, primordial follicle activation and viability as well as ovarian steroidogenesis. Thin arrows indicate positive regulation, negative regulation is indicated by the broken arrow. Block arrows indicate the impact of obesity.

Table 1

Sequences of primers used

Primer name	Sequence
Insr-for	ATGGGCTTCGGGAGAGGAT
Insr-rev	GGATGTCCATACCAGGGCAC
Irsl-for	GGATGTCCATACCAGGGCAC
Irsl-rev	CAGCCCGCTTGTTGATGTTG
Cyp2e1-for	CCCAAGTCTTTAACCAAGTTGGC
Cyp2e1-rev	CTTCCATGTGGGTCCATTATTGA
c-kit-for	GCCACGTCTCAGCCATCTG
c-kit-rev	GTCGGGATCAATGCACGTCA
Kitlg-for	GAATCTCCGAAGAGGCCAGAA
Kitlg-rev	GCTGCAACAGGGGGTAACAT
Akt1-for	CCACCTGTCTCTAGGGTCCA
Akt1-rev	CATGGGACACAGCAACAAAC
Gstp-for	CCCAAGTTTGAGGATGGAGA
Gstp-rev	CAGGGCCTTCACGTAGTCAT
Gstm-for	GAGAGGATCCGTGCAGACAT
Gstm-rev	ACTTGGGCTCAAACATACGG
Foxo3a-for	GGTACCAGGCTGAAGGATCA
Foxo3a-rev	CAGTCTCTGCTGGGTTAGGG